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(54) Title: METHODS OF *IN SITU* MODIFICATION OF PLANT GENES

(57) Abstract

A method of producing plants which exhibit an agronomically desirable trait comprises mutating or otherwise modifying *in situ* in a plant cell at least one gene which when modified is responsible for providing the said trait and regenerating from a cell exhibiting the said trait fertile morphologically normal whole plants, and is characterised in that a polynucleotide is introduced into the plant cell, the said polynucleotide comprising at least one region which is substantially complementary to at least one region in the gene, which gene region when mutated or otherwise modified provides for the agronomically desirable trait, the region in the said polynucleotide containing at least one base mismatch in comparison with the like region in the said gene, so that the region in the said gene is altered by the DNA repair/replication system of the cell to include the said mismatch.

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# METHODS OF IN SITU MODIFICATION OF PLANT GENES

The present invention relates to the production of plants which exhibit certain desirable agronomic traits and which are produced by a non-biological process not obligatorily involving transformation or transgenesis (although these techniques can be used).

According to the present invention there is provided a method of producing plants which exhibit an agronomically desirable trait comprising mutating or otherwise modifying *in situ* in a plant cell at least one gene which when modified is responsible for providing the said trait and regenerating from a cell exhibiting the said trait fertile morphologically normal whole plants, characterised in that a polynucleotide is introduced into the plant cell, the said polynucleotide comprising at least one region which is substantially complementary to at least one region in the gene, which gene region when mutated or otherwise modified provides for the agronomically desirable trait, the region in the said polynucleotide containing at least one base mismatch in comparison with the like region in the said gene, so that the region in the said gene is altered by the DNA repair/replication system of the cell to include the said mismatch.

By "gene" is meant a polynucleotide comprising - contiguously - a sequence to which an RNA polymerase is capable of binding (promoter), an RNA encoding sequence and a transcription termination sequence. At least one of the following regions of the gene may be mutated or otherwise modified: promoter, RNA encoding sequence or transcription terminator. In a preferred embodiment of the method a transcription enhancing region associated with the gene is mutated or otherwise modified *in situ*.

Whilst the said trait could be an improved resistance to insects and/or fungal or bacterial infections, it is particularly preferred that the trait is herbicide resistance. The herbicides to which plants resulting from the method according to the invention are rendered resistant, or to which the said plants are tolerant or exhibit relatively improved resistance, are selected from the group consisting of paraquat; glyphosate; glufosinate; photosystem II inhibiting herbicides; dinitroanalines or other tubulin binding herbicides; herbicides which inhibit imidazole glycerol phosphate dehydratase; herbicides which inhibit acetolactate synthase; herbicides which inhibit acetyl CoA carboxylase; herbicides which inhibit protoporphyrinogen oxidase; herbicides which inhibit phytoene desaturase; herbicides which

inhibit hydroxyphenylpyruvate dioxygenase and herbicides which inhibit the biosynthesis of cellulose.

Plants which are substantially "tolerant" to a herbicide when they are subjected to it provide a dose/response curve which is shifted to the right when compared with that provided by similarly subjected non tolerant like plants. Such dose/response curves have "dose" plotted on the x-axis and "percentage kill", "herbicidal effect" etc. plotted on the y-axis. Tolerant plants will require more herbicide than non tolerant like plants in order to produce a given herbicidal effect. Plants which are substantially "resistant" to the herbicide exhibit few, if any, necrotic, lytic, chlorotic or other lesions when subjected to the herbicide at concentrations and rates which are typically employed by the agrochemical community to kill weeds in the field. Plants which are resistant to a herbicide are also tolerant of the herbicide. The terms "resistant" and "tolerant" are to be construed as "tolerant and/or resistant" within the context of the present application.

The skilled man will appreciate that the plant material in which the *in situ* modification is performed may have been prior transformed with a gene providing for resistance to insects, fungi, and/or herbicides, or with a gene capable of providing plants regenerated from such material with, for example, an increased capacity to withstand adverse environmental conditions (improved drought and/or salt tolerance, for example) in comparison with plants regenerated from non-transformed like material.

At least one region of the polynucleotide may consist of RNA. The polynucleotide other than that comprised by the said at least one region may consist of DNA. The polynucleotide may consist of between about 30 and 250 nucleotides. In a more preferred embodiment of the polynucleotide it consists of between 50 and 200 nucleotides.

The protein encoding region of the gene may encode an enzyme selected from the group consisting of EPSPS, GOX, PAT, HPPD, ACC, ALS, BNX and protox and known mutated or variant forms thereof. In particular, the said gene may encode an EPSPS enzyme as depicted, for example, in SEQ ID Nos. 1 or 10. It is preferred that the EPSPS enzyme has least the residues Thr, Pro, Gly and Ala at positions corresponding to 174, 178, 173 and 264 with respect to the EPSPS depicted in SEQ ID No. 2, and that the said mismatch results in at least one of the following modifications in the EPSPS enzyme in comparison with the native sequence:

(i) Thr 174 - Ile

(ii) Pro 178 - Ser

(iii) Gly 173 - Ala

(iv) Ala 264 - Thr

wherein (i) Thr 174 occurs within a sequence comprising contiguously Ala -Gly-Thr-Ala-Met; (ii) Pro 178 occurs within a sequence comprising contiguously Met-Arg-Pro-Leu-Thr; (iii) Gly 173 occurs within a sequence comprising contiguously Asn-Ala-Gly-Thr-Ala; and (iv) Ala 264 occurs within a sequence comprising contiguously Pro-Leu-Ala-Leu-Gly.

Alternatively, and/or additionally, the mismatch may result in replacement of the terminal Gly residue within the sequence motif Glu-Arg-Pro-AA1-AA2-AA3-Leu-Val-AA4-AA5-Leu-AA6-AA7-AA8-Gly- in a region of the EPSPS enzyme corresponding to that spanning positions 202 to 216 in SEQ ID No. 2 by either an Asp or Asn residue.

The plant cell to which the method of the invention is applied may be a cell of a plant selected from the group consisting of canola, sunflower, tobacco, sugar beet, cotton, maize, wheat, barley, rice, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, cabbage, onion, soya spp, sugar cane, pea, field beans, poplar, grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax and oilseed rape, and nut producing plants insofar as they are not already specifically mentioned

The plant cell may be converted into a protoplast prior to the *in situ* mutation or modification of the gene - or transcriptional enhancing regions associated therewith - which when modified provides for the agronomically desirable trait.

The invention further includes plants which result from the method disclosed herein, as well as the progeny and seeds of such plants, and plant material derived from such plants, progeny and seeds.

The invention still further includes a method of selectively controlling weeds in a field, the field comprising plants as disclosed in the preceding paragraph and weeds, the method comprising application to the field of a herbicide to which the said plants have been rendered resistant. Insecticidally effective amounts of insecticides and/or fungicidally effective amounts of fungicides may optionally be applied to the said plants, preferably after the herbicide has been applied to the field.

The invention will be further apparent from the following description taken in conjunction with the associated sequence listing.

SEQ ID No. 1 shows the cDNA from Petunia encoding an EPSPS enzyme. Nucleotides 28 to 243 encode the transit peptide responsible for targeting the EPSPS enzyme encoded by nucleotides 244 to 1578 to the chloroplast. SEQ ID No. 2 shows the translational product of the sequence depicted in SEQ ID No. 1. Protein having the sequence of amino acid residues 1 to 72 constitutes the chloroplast transit peptide: protein having the sequence of amino acids 73 to 516 constitutes the EPSPS enzyme. SEQ ID Nos 3 and 4 depict peptides encoded by sequences (SEQ ID Nos 5 and 7) within exons 2 and 4 respectively of the *Brassica napus* EPSPS gene. Sequence ID Nos. 6 and 8 are mixed ribo-deoxyribonucleic acid sequences which are capable of forming duplexes with the sequences depicted in SEQ ID Nos. 5 and 7 respectively. SEQ ID Nos. 28 and 29 are sequences which are comprised by the sequences depicted in SEQ ID Nos. 5 and 7 respectively. SEQ ID Nos. 9 and 10 depict respectively (i) the genomic DNA *from Brassica napus* which encodes a spliced RNA encoding an EPSPS enzyme, and (ii) the amino acid sequence of the said *Brassica* EPSPS enzyme. SEQ ID Nos 11 - 27 depict mixed oligonucleotides (ie containing both ribo and deoxyribonucleotides) comprising sequences (marked with asterixes in the reiteration of the sequences in the corresponding Examples) capable of causing mutations in the gene to which the oligonucleotide is targeted. The oligonucleotides depicted in SEQ ID Nos 11 to 27 are all designed to cause plant material into which they are incorporated to become resistant to herbicides, such as glyphosate and chlorsulfuron, by causing the gene encoding the proteinaceous target for the herbicide to become mutated so that the target is no longer sensitive to the herbicide. Should there by a discrepancy between the sequences depicted in the sequence listings and those corresponding sequences depicted in the Examples, the Example sequences are definitive. In the Examples sequences depicted in lower case are RNA and those in upper case are DNA.

Methods

Polynucleotides Mixed ribo-deoxyribonucleic acids are synthesised by synthetic and semisynthetic methods known to those skilled in the art (for example Scaringe, S.A. et al (1990), Nucleic Acids Research 18:5433-5441; Usman, N. et al (1992) Nucleic Acids Research 20:665-6699 and Swiderski, P.M. et al (1994) Anal. Biochem. 216:83-88. Eric B. Kmiec (1996) United States Patent 5,565,350). Mixed ribo-deoxyribonucleic acids are synthesised using natural nucleotides, or, in some cases, preferably with 2'-O methylated ribonucleotides. Additionally or alternatively the phosphodiester bonds of the nucleic acid

are replaced by phosphorothiodiesters or methylphosphonodiesters. Additionally or alternatively arabinose-containing nucleotides are also used.

A duplex nucleic acid in which deoxyribonucleotides and ribonucleotides correspond with each other is termed a hybrid-duplex. When two strands form a region of duplex nucleic acid for less than all of their bases the resultant molecule is termed a heteroduplex. Two strands of a duplex can be linked by an oligonucleotide linker region to form a single polymer. The bases in the linker region are not Watson-Crick paired. A heteroduplex in which the first and second strands are portions of a single polymer is termed a hairpin duplex.

The mixed ribo-deoxyribonucleic acid useful in the present invention has at most one 3' end and one 5' end. It is constructed to contain at least one region of at least one or more - usually three to four - bases that are not Watson-Crick paired. These unpaired regions form linker regions between two strands of Watson-Crick paired bases. It is preferred that the bases of the linker regions are deoxyribonucleotides.

In a preferred embodiment, the mixed ribo-deoxyribonucleic acid is constructed having two linkers arranged a) such that substantially all of the remaining bases are Watson-Crick paired and b) such that the 3' and 5' ends of the polymer are Watson-Crick paired to adjacent nucleotides of the complementary strand. These can be ligated to form a single continuous circular mixed ribo-deoxyribonucleic acid polymer.

In the present invention, the mixed ribo-deoxyribonucleic acid is used for the purpose of specifically introducing alterations (a mutation) into a target gene. The genetic site of alteration is determined by selecting a portion of the mixed ribo-deoxyribonucleic acid to have the same sequence as (to be homologous with) the sequence of the target site, hereinafter termed a homologous region. The area of differences between the sequence of the mixed ribo-deoxyribonucleic acid and the target gene is termed the heterologous region. Preferably there are two homologous regions in each mixed ribo-deoxyribonucleic acid flanking an interposed heterologous region, all three regions being present in a single continuous duplex nucleic acid. Furthermore each homologous region contains a portion of hybrid duplex nucleic acid. The portion of each hybrid-duplex is at least 4 base pairs, preferably 8 base pairs and more preferably about 20 to 30 base pairs. A dinucleotide base pair of homo-duplex may be placed within a region of hybrid duplex to allow ligation of the

3' and 5' ends to each other. The total length of the two homologous regions is at least 20 base pairs and preferably is between 40 and 60 base pairs.

A region of homo-duplex can be disposed between the hybrid-duplex/ homologous regions of the vector. The interposed homo-duplex can contain the heterologous region. When the heterologous region is less than about 50 base pairs and preferably less than about 20 base pairs, the presence of an interposed homo-duplex is optional. When the heterologous region exceeds about 20 base pairs, an interposed homo-duplex is preferred.

The change to be introduced into the target gene is encoded by the heterologous region. The change to be introduced may be a change in one or more bases of the target gene sequence or the addition of one or more bases.

Design of polynucleotides to achieve in situ mutagenesis of EPSP synthase in Brassica napus variety Westar. It is known that the combination of mutations G101A and A192T in a Petunia EPSPS can provide for resistance to glyphosate, whilst maintaining a low Km for PEP. The equivalent residues in the sequence of the *B.napus* enzyme are (1) the second glycine occurring within the sequence LGNAGTAMRPLT (SEQ ID No. 3) where this G is amino acid 173 wherein amino acid 1 is the starting methionine of the transit peptide and (2) the third alanine occurring within the sequence MAAPLALGDVEI (SEQ ID No. 4) and consequential having the residue number 264.

The glycine residue occurs within exon 2 (part of which is shown below and is depicted as SEQ ID No. 5), the DNA coding sequence in the region being:

L G N A G T A M R P L T

ATTGAGTTGTACCTTGGGAATGCAGGAACAGCCATGCGTCCACTCACCGCTGCA

An example of the desired mutation is GGA ---> GCA

The mixed ribo-deoxyribonucleic acid designed to elicit this change includes, for example, on one of its strands, a sequence comprising mainly of RNA which is complementary to all or part of the above DNA sequence. This RNA is interposed by a short region of DNA also complementary with the corresponding region of the above DNA sequence except for the inclusion of the specific mismatch of having a guanosine base opposite the guanosine base within the target GGA codon. A suitable mixed ribo-deoxyribonucleic acid could thus include all or part of the following sequence (depicted as SEQ ID No. 6 in the sequence listing). Note that RNA sequence is marked in bold.

TTGTACCTTGGGAATGCAGGAACAGCCATGCGTCCACTC

**AACAUGGAACCCUUACG**TCGTT**GUCGGUACGCAGGUGAG**

The corresponding alanine residue occurs within exon 4 (part of which is shown below and is depicted as SEQ ID No. 7).

M A A P L A L G D V E I

ACTGCCCTCCTCATGGCAGCTCCTTTAGCTCTTGGAGACGTGGAGATTGAGATCATT

An example of the desired mutation is GCT ---> ACT. The mixed ribo-deoxyribonucleic acid designed to elicit this change includes, for example, on one of its strands, a sequence comprising mainly of RNA which is complementary to all or part of the above DNA sequence. This RNA is interposed by a short region of DNA also complementary with the corresponding region of the above DNA sequence except for the inclusion of the specific mismatch of having a thymine base opposite the guanosine base within the target GCT codon. The desired polynucleotide thus includes all or part of the RNA sequence depicted below and in SEQ ID No. 8. Note that RNA sequence is marked in bold.

TCCTCATGGCAGCTCCTTTAGCTCTTGGAGACGTGGAGATT

**AGGAGUACCGUCGAGGAA**ATTGA**GAACCUCUGCACCUCUAA**

Besides the examples detailed above there will of course be many other specific changes which could be introduced into those sequences which regulate gene expression and for which polynucleotides can easily be designed by methods directly analogous to that described above and which, for example, could be useful to achieve increased expression of EPSPS. The skilled man will appreciate that many methods could be used to specify those changes potentially useful for increasing the expression of EPSPS. For example:

(1) The skilled man will be aware of instances of resistance to glyphosate having occurred in both field populations of weeds (e.g Australian lolium) and upon continuous selection of cultured plant cells (e.g. Hollander-Czytko et al (1988) in Plant Mol. Biol, 11, 215-220; Hollander-Czytko et al (1992) Plant. Mol. Biol. 20, 1029-1036) or, for example, cultivars of birdsfoot trefoil (Boerboom et al (1990) Weed. Sci., 38, 463-467) upon glyphosate. In the latter two cases selection was shown to have resulted in a significant increase in expression of EPSP synthase. In the example of the work on cell cultures of Corydalis sempervirens (Hollander-Czytko et al (1988) in Plant Mol. Biol, 11, 215-220) a 30-40 fold increase in the cellular content of EPSP synthase and an 8-12 fold increase in transcript levels was observed. There was no amplification of the EPSP synthase gene.

It is a routine matter in all of the above examples using methods known to the skilled man to isolate cDNA encoding the EPSP synthases, to use these cDNA's as probes to identify clones from genomic libraries and to sequence the corresponding EPSP synthase genes and their 5' upstream and 3' downstream regions. Alternatively, genomic sequences may be isolated directly using heterologous probes and/or combinations of degenerate and inverse PCR. By comparing the sequences so obtained from 'high EPSP synthase expression' lines of plants, cultivars or plant cells with the appropriate unselected controls the specific mutation(s) responsible for conferring high expression of EPSP synthase will be identified.

(2) Another example of a suitable method for identifying mutations potentially useful for increasing the expression of EPSP synthase is to directly select various lines of cultured plant cells or protoplasts from plant species of interest (e.g. *Brassica napus*) on increasing concentrations of glyphosate. This can be done with or without the addition of a suitable chemical mutagen. Glyphosate-tolerant lines so obtained are analysed for expression of EPSP synthase, for the level of translatable EPSP synthase gene transcript (e.g by Northern analysis) and for possible amplification of the EPSPS gene (e.g. by Southern and dot blot analysis). Cell lines of particular interest would be those where EPSP synthase was overexpressed and where this increase could not be accounted for through gene amplification. Identification of the specific mutation(s) responsible for conferring high expression of EPSP synthase are then identified as described in (1) above.

(3) A further example of a method useful to specify mutations causing high expression of EPSPS comprises (a) subcloning the plant EPSP synthase promoter, 5' upstream sequence region, translational start region and sequence encoding the N-terminus region of EPSP synthase into a translational fusion construct directing the synthesis of a suitable and easily measurable reporter gene such as (Beta glucuronidase) (b) further cloning this into a shuttle vector containing an origin for replication in *E.coli* and also designed for site specific integration into the yeast genome (YIP), or the genome of any other suitable test cell, such that integration into a specific location can be positively selected, by for example, complementation of an auxotrophic mutation. A library of many variants specifically within the promoter and 5' upstream region of the so-designed shuttle vector is then created by mutagenesis through, for example, Mn2+-poisoned PCR of the region and maintained in *E.coli*. Members of the library are then tested by transformation into yeast. The best

expressers in yeast are identified by increased expression of the reporter gene. The integrated DNA from these high expresser lines is then extracted, sequenced and compared with the original sequence in order to identify those specific mutation(s) which conferred increased expression. Such mutations may affect conserved domains within the promoter which bind the transcriptional activators required for gene expression. Studies of this sort may teach those skilled in the art to modify the equivalent conserved regions in other crop plant species, thus enabling the technology to be applied broadly.

The polynucleotides comprising the RNA sequences disclosed above are transfected into protoplasts of *Brassica napus* which are then cultured and subjected to the herbicide glyphosate at concentrations which are sufficient to kill like protoplasts which have not been transfected and like protoplasts which have been transfected but with a polynucleotide not comprising regions designed to elicit a mutation in the *Brassica* genome. Those transfected protoplasts which survive the herbicide at concentrations which kill the control protoplasts are regenerated into plants using known means. The increased resistance to the herbicide of the thus regenerated plants is inherited in a Mendelian manner amongst the progeny of these plants.

The skilled man will appreciate that the invention is not limited to that specifically described above in respect of the production of glyphosate resistant *Brassica napus*. For plant species for which the EPSP synthase gene sequence(s) are already available on public databases the RNA and DNA elements of the polynucleotides can easily be designed by a method directly analogous to that described for *B. napus*. Polynucleotides comprising these RNA and DNA elements can then be introduced into regeneratable plant material from other species. Moreover, the skilled man is capable of designing:

(i) polynucleotides for the *in situ* mutagenesis of the DNA bases flanking the translational start site to improve post transcriptional efficiency of expression of EPSP synthase in plants, for example *Brassica napus variety Westar*. The consensus sequences for the regions immediately surrounding the translational start sites in animals (M Kozak, 1986, Cell, 44, 283-292) and plants (G Heidecker and J Messing, 1986, Ann. Rev. Plant Physiol.. 37, 439-466; V Pautot et al., 1989, Gene, 77, 133-140) have been described. It is therefore possible that improved levels of expression of the native B. napus EPSP synthase gene may be improved in situ by designing mixed ribo-deoxyribonucleic oligonucleotides to make the

desired mutational changes, at positions -3 and + 6 as shown below. Note that conserved consensus sequences are underlined.

| | -4 | -3 | -2 | -1 | +1 | +2 | +3 | +4 | +5 | +6 |
|---|---|---|---|---|---|---|---|---|---|---|
| B. napus | A | T | C | A | A | T | G | G | C | G |
| Concensus | A | A | C | A | A | T | G | G | C | T |

It will be obvious to those skilled in the art that this approach need not be confined to the EPSP synthase gene from B. napus, but may be applied to any plant species in which an increase in expression of the target gene is sought.

ii) polynucleotides for the *in situ* mutagenesis of the DNA bases to achieve an increase in transcriptional efficiency of expression of EPSP synthase. An approach similar to that described above may be adopted to achieve an enhancement in the rate of transcription of EPSP synthase genes by mutating bases at the "TATA" box region upstream from the transcription start point, and at the transcription start point itself. Identification of the transcription start point is identified using techniques, such as primer extension analysis, known to those skilled in the art. The "TATA" box is generally found 16-54 bases upstream of the transcriptional start. Consensus sequences have been published for plant transcription start point (V Pautot et al., 1989, Gene, 77, 133-140)

Plant Consensus CTCATCA

and "TATA" box regions (V Pautot et al., 1989, Gene, 77, 133-140)

Plant Consensus TCACTATATATAG

In both cases highly conserved bases are underlined. Comparisons between the consensus and native sequences of target EPSP synthase genes will enable bases suitable for mutational change to be identified.

(iii) polynucleotides for *in situ* mutagenesis to alter expression of EPSP synthase in plants, for example *Brassica napus variety Westar.*

Such designed polynucleotides can be introduced into totipotent plant material by known means which is then regenerated into plants which are subjected to a selection procedure to isolate those that exhibit the desired trait.

The skilled man will appreciate that directly analogous methods to those described above for EPSP synthase and glyphosate could be applied to other combinations of selecting herbicide and target gene where the aim is to specify mutations conferring over-expression.

The invention will be further apparent from the following Examples. Throughout the Examples the expression "selecting concentrations" of herbicide is present. By this is meant a concentration of herbicide which is sufficient to kill non-transformed material, or material which otherwise does not contain the oligonucleotides which are contained within like experimental material. The skilled man will know what those concentrations are having regard to the specific circumstances relating to his particular germplasm, transformation protocols and the expected variation between replicate procedures. The oligonucleotides shown below (SEQ ID Nos 11 to 27) are all synthesised according to Yoon *et al.* (1996). In each of the Examples where the constructs contain bases depicted in lower case, the sequence comprising such bases is to be understood as being RNA, and sequences comprising bases depicted in upper case as being DNA.

**Example 1** This Example demonstrates the production of corn (maize) which is resistant to the herbicide chlorsulfuron.

```
                    *
 TGCGCG gauacuagggATTACcaccccgaaT
T                                  T
T                                  T
 TCGCGC CTATGATCCCTAATGGTGGGGCTTT
      3'5'
```

The above oligonucleotide (SEQ ID No. 11) conveniently may be introduced into corn using silicon carbide whiskers, pollen harbouring the oligonucleotide or *via* pollen tubes.

**Whiskers** The so called whiskers technique is performed essentially as described by Frame *et al.*, (1994 Plant J. 6 941 -948). The oligonucleotide (1-100 µg) depicted in SEQ ID No.11 is added to the whiskers and used to transform A188 x b73 cell suspensions. The oligonucleotide(s) may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. Plant regeneration is performed using selective concentrations of chlorsulfuron in place of bialophos. Plants are transferred to pots and matured in the green house. Kernals from these plants are germinated in soil and sprayed with a selecting concentration of chlorsulfuron 9 to 14 days post emergence.

**Pollen transformation** Maize pollen is bombarded with gold particles by techniques known to the skilled man. Gold particles are coated with the oligonucleotide depicted in SEQ ID No. 11. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. Suitable bombardment methods vary in precise detail but the basic procedure is well known to the skilled man and it is thus not necessary to describe it here. Bombarded pollen is applied to receptive silks of detassled plants. Sufficient replicas are performed to pollinate a large number of plants (typically up to 500). Progeny of the plants are screened for chlorsulfuron resistant members of the population by spraying with selecting concentrations of chlorsulfuron.

**Pollen tube mediated transformation** Emasculated corn plants are used. Wild type pollen is applied to pollination receptive silks. After between 30 min to 6 hours the silks are cut to within one cm of the base. The above SEQ ID No. 11 oligonucleotide (1-100 μg/ 10 μl in TE) is applied to the cut surface using a 1 ml syringe and needle such that the surface is completely covered. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. The plants are then grown in a green house with an initial humidity of about 75 %. Progeny of the plants are screened for chlorsulfuron resistant members of the population by spraying with selecting concentrations of the herbicide.

Plants derived from material into which the oligonucleotides have been incorporated are resistant, more resistant or tolerant to the herbicide, when compared to plants derived from material not containing the said oligonucleotide.

**Example 2** This Example demonstrates the production of *Arabidopsis thaliana* which is resistant to the herbicide glyphosate (and suitable salts thereof). The following oligonucleotides (depicted as SEQ ID Nos 12 to 16 in the sequence listing) are prepared using standard technology.

T to I

```
                    *
 T GCGCG cauuacguccTTATCguuacgcagg T
T                                   T
T                                   T
 T CGCGC GTAATGCAGGAATAGCAATGCGTCC T
       3'5'     (SEQ ID No. 12)
```

T to I 2

```
                    *
 T GCGCG cauuacgtccTTATCguuacgcaag T
T                                   T
T                                   T
 T CGCGC GTAATGCAGGAATAGCAATGCGTTC T
       3'5'     (SEQ ID No. 13)
```

P to S

```
                    *
 T GCGCG ugucguuacgCAAGTgaauggcgac T
T                                   T
T                                   T
 T CGCGC ACAGCAATGCGTTCACTTACCGCTG T
       3'5'     (SEQ ID No. 14)
```

P to S 2

```
                    *
 T GCGCG uaucguuacgCAAGTgaauggcgac T
T                                   T
T                                   T
 T CGCGC ATAGCAATGCGTTCACTTACCGCTG T
       3'5'     (SEQ ID No. 15)
```

```
                    *       *
 T GCGCG cauuacguccTTATCguuacgCAAGTgaguggcgac T
T                                               T
T                                               T
 T CGCGC GTAATGCAGGAATAGCAATGCGTTCACTCACCGCTG T
       3'5'     (SEQ ID No. 16)
```

These oligonucleotides are introduced into *Arabidopsis* by microprojectile bombardment or protoplast uptake.

**Bombardments** *Arabidopsis* is transformed essentially using a modified procedure as described by Seki *et al.* ((1991) Appl. Microbiol. Biotechnol. 36 228-230). *Arabidopsis thaliana* genotype C24 seeds are surface sterilised and sown on B-5 medium

(Gamborg *et al* ., 1968) solidified with 0.6 % agarose. The plants are grown aseptically for 4 - 6 weeks under 16 h light 8 h dark at 26 °C. Roots are harvested and cut into sections that are 0.5 - 1.0 cm long and placed onto a filter paper on medium containing B5 salts and vitamins, 3 % sucrose, 0.5 mg/ml 2,4-dichloropheonoxyacetic acid, 0.05 mg/l kinetin and 0.8 % agarose (0.5 - 0.05 medium). After two to five days the roots are ready for bombardment. Gold particles (10 mg; Hereus, 0.4-1.2 um diameter) are coated with 1 - 100 µg of oligonucleotide as follows. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. The particles are suspended in 1 ml of absolute ethanol and incubated for three hours at room temperature then stored at -20oc. Twenty to thirty-five µl of sterile resuspended particles are collected by centrifugation in a microcentrifuge. The particles are washed with one ml of sterile distilled water and re-collected by centrifugation. Microprojectiles are then resuspended in 30 µl oligonucleotide solution (1 -100 µg), 25 µl of 1M CaCl2 is added followed by 10 µl of 0.1 M spermidine (free base). The mixture is incubated on ice for 10 minutes. 1 -10 µl of this solution is used per bombardment. A suitable mixture or combination of oligonucleotides is introduced into plant material either simultaneously or sequentially. If the oligonucleotides are introduced sequentially, they must be introduced in such a way that the mutation governed by the first oligonucleotide is not negated by the mutation governed by a subsequently introduced oligonucleotide. For example, if the oligonucleotide depicted by SEQ ID No. 12 is introduced first, the oligonucleotide depicted by SEQ ID No. 15 should be used subsequently. Alternatively, a single oligonucleotide comprising regions providing for multiple mutations (such as that depicted in SEQ ID No. 16) may be used.

The roots are bombarded with oligonucleotide-coated particles by a helium-driven biolistics PDS 1000 system (BioRad) with a 300 mm Hg vacuum. The levels between the rupture disk and the macrocarrier and the macro-carrier and sample are varied for maximal transformation efficiency. Rupture disks of between 1000 and 2000 psi are used. Two suitable oligonucleotides are introduced into *Arabidopsis* plant material either simultaneously or sequentially. For simultaneous transformation the oligonucleotides are used in equal molar concentrations and may be introduced into the material by multiple firings into the same tissue. For sequential transformation the roots receive at least one

bombardment with each oligonucleotide but multiple firings of each oligonucleotide are used if necessary to optimise transformation efficiencies.

After the bombardments the plant material is transferred to 0.5 - 0.05 medium and incubated at 26oc for one to 5 days. Regeneration of transformed material into *Arabidopsis* plants is performed as Seki *et al* 1991 with the exception that kanamycin or gentamycin are not included in any of the media. Instead the transformed material is selected by its resistance or tolerance to glyphosate, present in the selection medium at a concentration sufficient to kill control material which has been subjected to a like transformation procedure with the *proviso* that it does not contain the oligonucleotides specified above.

**DNA uptake by protoplasts incubated in PEG** The protocol of Dam *et al.* (1989 Mol Gen. Genet 217 6-12) is followed. Instead of using linearised plasmid DNA in the transformation an equal molar ratio mix of the two oligonucleotides (SEQ ID Nos 12 and 15) are used (1- 100 μg) with 50 -100 μg calf thymus carrier DNA. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. Glyphosate selection instead of hygromycin selection is applied at the same stage during callus formation. The concentration of glyphosate used is varied to give optimum selection of transformed *Arabidopsis* plants, but is determined by reference to suitable control experiments.

Plants derived from material into which the oligonucleotides have been incorporated are resistant, more resistant or tolerant to the herbicide, when compared to plants derived from material not containing the said oligonucleotide.

**Example** 3 This Example demonstrates the provision of glyphosate resistant *Brassica napus*

T to I

```
                    *
 T GCGCG ccuuacguccTTATCgcuacgcagg T
T                                  T
T                                  T
 T CGCGC GGAATGCAGGAATAGCCATGCGTCC T
      3'5' (SEQ ID No. 17)
```

T to I 2

```
                        *
 T GCGCG ccuuacgtccTTATCgcuacgcaag T
T                                   T
T                                   T
 T CGCGC GGAATGCAGGAATAGCCATGCGTTC T
       3'5'    (SEQ ID No. 18)
```

P to S

```
                       *
 T GCGCG ugucgguacgCAAGTgaguggcgac T
T                                   T
T                                   T
 T CGCGC ACAGCCATGCGTTCACTCACCGCTG T
       3'5'    (SEQ ID No. 19)
```

P to S 2

```
                       *
 T GCGCG uaucgguacgCAAGTgaguggcgac T
T                                   T
T                                   T
 T CGCGC ATAGCCATGCGTTCACTCACCGCTG T
       3'5'    (SEQ ID No. 20)
```

```
                       *          *
 T GCGCG ccuuacguccTTATCgcuacgCAAGTgaguggcgac T
T                                              T
T                                              T
 T CGCGC GGAATGCAGGAATAGCCATGCGTTCACTCACCGCTG T
       3'5'    (SEQ ID No. 21)
```

These oligonucleotides are designed to target the *Brassica napus* EPSPS gene. The oligonucleotides provide for two changes in the sequence of the protein encoded by the gene, *viz.* at T102 and P106 of the Brassica mature enzyme such that the mutant gene (*via* an altered protein product) confers resistance to glyphosate.

The oligonucleotides are introduced into *Brassica napus* using known methods which includes microprojectile bombardment or uptake of DNA by protoplasts.

**Bombardments** Seeds of *B.napus cv Westar* are surface sterilised in 1% sodium hypochlorite for 20 minutes. The seeds are then washed in sterile water three times and planted at a density of about 10 seeds per plate on Murashige Skoog (MS) minimal organics medium (GibcoBrl) with 3% sucrose and 0.7% phytagar (Gibco) pH 5.8. Seeds are germinated at 24 °C in 16 h light/8h dark. After five days the cotyledons are excised in such a

way that they include approximately 2 mm of petiole at the base. Care is taken to exclude the apical meristem. The excised cotyledons are placed on MS medium, 3 % sucrose and 0.7 % phytagar enriched with 20 μM bezyladenine with the petioles imbedded to a depth of 2 mm in the medium at a density of about ten cotyledons per plate.

Gold particles (10 mg; Hereus, 0.4-1.2 um diameter) are coated with 1 - 100 μg of oligonucleotide (SEQ ID No. 22 for example, or SEQ ID Nos. 18 and 20) in plant cells. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. The particles are suspended in 1 ml of absolute ethanol and incubated for three hours at room temperature then stored at -20oc. Twenty to thirty five μl of sterile resuspended particles are collected by centrifugation in a microcentrifuge. The particles are washed with one ml of sterile distilled water and re-collected by centrifugation. Microprojectiles are then resuspended in 30 μl solution (containing oligonucleotides depicted in SEQ ID Nos. 18 and 20, for example in an amount of about 1 -100 ug). 25 μl of 1M CaCl2 is added followed by 10 μl of 0.1 M spermidine (free base). The mixture is incubated on ice for 10 minutes. 1 -10 μl of this solution is used per bombardment.

The cotyledons are bombarded with oligonucleotide-coated particles by a helium-driven biolistics PDS 1000 system (BioRad) with a 300 mm Hg vacuum. The levels between the rupture disk and the macrocarrier and the macro-carrier and sample are varied for maximal transformation efficiency. Rupture disks of between 1000 and 2000 psi are used. The two oligonucleotides are introduced into the *Brassica* plant material either simultaneously or sequentially. For simultaneous transformation the oligonucleotides are used in equal molar concentrations and may be introduced into the explant by multiple firings into the same tissue. For sequential transformation the explants receive at least one bombardment with each oligonucleotide but multiple firings of each oligonucleotide are used as necessary to optimise transformation efficiencies.

After bombardment the explants are placed onto regeneration medium comprising MS medium supplemented with 20 μM benzyladenine, 3% sucrose 0.7% phytagar pH 5.8. After 2 - 5 days the cotyledons are transferred to plates containing the same media but including selective concentrations of glyphosate. The petioles remain embedded in the media. The explants are left for 2 - 6 weeks and then transferred onto MS medium

supplemented with 3 % sucrose, 0.7% phytagar pH 5.8 and selecting concentrations of glyphosate. One to three weeks later surviving shoots are transferred to rooting media which comprises MS medium, 3% sucrose, 2 mg/ml indole butyric acid, 0.7% phytagar with no glyphosate. Once roots are visible the plants are transferred to pots and propagated in the greenhouse.

**Protoplast uptake** The method of Golz *et al.* ((1990) Plant Mol Biol 15 475 - 483) is followed. *Brassica napus* genotype H1 is used. Instead of using plasmid DNA in the transformation an equal molar ratio mix of the two oligonucleotides (SEQ ID Nos 18 and 20) are used (1- 100 μg) and 20 -100 μg calf thymus carrier DNA. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. Glyphosate selection instead of hygromycin selection is applied at the same stage during callus formation. The concentration of glyphosate used is varied to give optimum selection of transformed *Brassica* plants.

Plants derived from material into which the oligonucleotides have been incorporated are resistant, more resistant or tolerant to the herbicide, when compared to plants derived from material not containing the said oligonucleotide.

**Example 4** This Example demonstrates the provision of corn resistant to the herbicide glyphosate (and salts thereof).

T to I

```
                    **
 T GCGCG ccuuacgaccTTAGCGuuacgccggua T
T                                    T
T                                    T
 T CGCGC GGAATGCTGGAATCGCAATGCGGCCAT T
      3'5'    (SEQ ID No. 22)

                    **
 T GCGCG ccuuacgaccTTAGCGuuacgccagua T
T                                    T
T                                    T
 T CGCGC GGAATGCTGGAATCGCAATGCGGTCAT T
      3'5'    (SEQ ID No. 23)
```

```
P to S
                    *
 T GCGCG gacguuacgCCAGTaacugucgucg T
T                                   T
T                                   T
 T CGCGC CTGCAATGCGGTCATTGACAGCAGC T
      3'5'     (SEQ ID No. 24)

P to S 2
                   *
 T GCGCG agcguuacgCCAGTaacugtcgucg T
T                                   T
T                                   T
 T CGCGC TCGCAATGCGGTCATTGACAGCAGC T
      3'5'     (SEQ ID No. 25)


                      **        *
 T GCGCG ccuuacgaccTTAGCGuuacgCCAGTaacugucgucg T
T                                              T
T                                              T
 T CGCGC GGAATGCTGGAATCGCAATGCGGTCATTGACAGCAGC T
      3'5'     (SEQ ID No. 26)
```

These oligonucleotides which are designated as SEQ ID Nos 22-26 in the sequence listing and which are produced by means known to the skilled man, may be introduced into corn using silicon carbide whiskers, pollen harbouring oligonucleotides or via pollen tubes.

**Silicon carbide whiskers** This transformation is performed essentially as described by Frame *et al.* (1994 Plant J. 6 941-948). The oligonucleotide depicted as SEQ ID No 26 (1-100 μg) is added to the whiskers and used to transform A188 x B73 cell suspensions. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. Plant regeneration is performed using selective concentrations of glyphosate in place of bialophos. Plants are transferred to pots and are then matured in the green house. Caryopsis from these plants are germinated in soil and sprayed with a selecting concentration of glyphosate 9 to 14 days post emergence.

**Pollen transformation.** Maize pollen is bombarded with gold particles (essentially as described in the above Examples) coated with a mixture of the above oligonucleotides (SEQ ID Nos 23 and 25). The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such

that recombination is catalysed between the oligonucleotide and the target sequence. Bombarded pollen is applied to receptive silks of detassled plants. Sufficient replicas are performed to pollinate a large number (typically up to 300) of plants. Progeny of the plants are screened for glyphosate resistant members of the population by spraying with selecting concentrations of glyphosate.

**Pollen tube mediated transformation** Emasculated corn plants are used. Wild type pollen is applied to pollination receptive silks. After between 30 min to 6 hours the silks are cut to within one cm of the base. Suitable mixtures of the above oligonucleotides (1-100μg/ 10 μl in TE) are applied to the cut surface using a 1 ml syringe and needle such that surface is completely covered. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. The plants are then grown in a green house with an initial humidity of about 75 %. Progeny of the plants are screened for glyphosate resistant members of the population by spraying with selecting concentrations of glyphosate.

Plants derived from material into which the oligonucleotides have been incorporated are resistant, more resistant or tolerant to the herbicide, when compared to plants derived from material not containing the said oligonucleotide.

**Example** 5 This Example demonstrates the provision of tomato plants resistant to a bleaching herbicide designated as R390244.

```
                    *
 T GCGCC agcguaacuuGTCGAaagaagucca T
T                                   T
T                                   T
 T CGCGC TCGCATTGAACAGCTTTCTTCAGGT T
      3'5'     (SEQ ID No. 27)
```

This oligonucleotide (SEQ ID No. 27) is designed to target the codon for arginine 307 of the tomato phytoene desaturase (PDS) gene and introduce a mutation such that the mutant PDS is resistant to the herbicide R390244. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. The oligonucleotide is introduced into tomato Mill cv H722 *via* microprojectile bombardment essentially as described by Eck *et al.* (1995 Plant Cell Reports

14, 299-304) and as outlined above for the other crops subjected to this transformation procedure.

Regenerable cotyledon explant material (as described by Fillati *et al.* (1997 Bio/technology 5 726-730) suspensions are bombarded with SEQ ID No. C oligonucleotide-coated particles by a helium-driven biolistics PDS 1000 system (BioRad) with a 300 mm Hg vacuum. The levels between the rupture disk and the macrocarrier and the macro-carrier and sample are varied for maximal transformation efficiency. Rupture disks of between 1000 and 2000 psi are used. The oligonucleotide may be introduced into the explant by multiple firings into the same tissue as necessary to optimise transformation efficiencies. The regenerable cotyledons are bombarded at the same stage as when *Agrobacterium* is used in the method of Beaudoin and Rothstein (1997 Plant Mol Biol 33 835 -846). Regeneration of tomato plants is as described by Beaudoin and Rothstein except that no selection agent is used. Primary putative transformants are grown in the greenhouse and cuttings are propagated in soil. These cuttings, once established, are sprayed with selecting concentrations of R390244 and allow transformed herbicide resistant plants to be identified. These transformed plants are grown to maturity and seeds resulting from self pollination are collected.

Mutation events in individuals is confirmed by amplifying the particular mutant gene sequence from herbicide resistant individuals spanning the region of mutation by PCR and sequencing individually isolated and cloned sequences.

Plants derived from material into which the oligonucleotides have been incorporated are resistant, more resistant or tolerant to the herbicide, when compared to plants derived from material not containing the said oligonucleotide.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ZENECA LTD
(B) STREET: 15 Stanhope Gate
(C) CITY: LONDON
(E) COUNTRY: GB
(F) POSTAL CODE (ZIP): W1Y 6LN

(ii) TITLE OF INVENTION: IMPROVEMENTS IN OR RELATING TO ORGANIC COMPOUNDS

(iii) NUMBER OF SEQUENCES: 29

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1944 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Petunia hybrida

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:28..1578

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
GAATTCCCTC AATCTTTACT TCAAGA ATG GCA CAA ATT AAC AAC ATG GCT          51
                             Met Ala Gln Ile Asn Asn Met Ala
                               1               5

CAA GGG ATA CAA ACC CTT AAT CCC AAT TCC AAT TTC CAT AAA CCC CAA       99
Gln Gly Ile Gln Thr Leu Asn Pro Asn Ser Asn Phe His Lys Pro Gln
     10                  15                  20

GTT CCT AAA TCT TCA AGT TTT CTT GTT TTT GGA TCT AAA AAA CTG AAA      147
Val Pro Lys Ser Ser Ser Phe Leu Val Phe Gly Ser Lys Lys Leu Lys
 25                  30                  35                  40

AAT TCA GCA AAT TCT ATG TTG GTT TTG AAA AAA GAT TCA ATT TTT ATG      195
Asn Ser Ala Asn Ser Met Leu Val Leu Lys Lys Asp Ser Ile Phe Met
                 45                  50                  55

CAA AAG TTT TGT TCC TTT AGG ATT TCA GCA TCA GTG GCT ACA GCA CAG      243
Gln Lys Phe Cys Ser Phe Arg Ile Ser Ala Ser Val Ala Thr Ala Gln
             60                  65                  70
```

```
AAG CCT TCT GAG ATA GTG TTG CAA CCC ATT AAA GAG ATT TCA GGC ACT      291
Lys Pro Ser Glu Ile Val Leu Gln Pro Ile Lys Glu Ile Ser Gly Thr
          75                  80                  85

GTT AAA TTG CCT GGC TCT AAA TCA TTA TCT AAT AGA ATT CTC CTT CTT      339
Val Lys Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu Leu
      90                  95                 100

GCT GCC TTA TCT GAA GGA ACA ACT GTG GTT GAC AAT TTA CTA AGT AGT      387
Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Ser Ser
 105                 110                 115                 120

GAT GAT ATT CAT TAC ATG CTT GGT GCC TTG AAA ACA CTT GGA CTG CAT      435
Asp Asp Ile His Tyr Met Leu Gly Ala Leu Lys Thr Leu Gly Leu His
                 125                 130                 135

GTA GAA GAA GAT AGT GCA AAC CAA CGA GCT GTT GTT GAA GGT TGT GGT      483
Val Glu Glu Asp Ser Ala Asn Gln Arg Ala Val Val Glu Gly Cys Gly
             140                 145                 150

GGG CTT TTC CCT GTT GGT AAA GAG TCC AAG GAA GAA ATT CAA CTG TTC      531
Gly Leu Phe Pro Val Gly Lys Glu Ser Lys Glu Glu Ile Gln Leu Phe
         155                 160                 165

CTT GGA AAT GCA GGA ACA GCA ATG CGG CCA CTA ACA GCA GCA GTT ACT      579
Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val Thr
     170                 175                 180

GTA GCT GGT GGA AAT TCA AGG TAT GTA CTT GAT GGA GTT CCT CGA ATG      627
Val Ala Gly Gly Asn Ser Arg Tyr Val Leu Asp Gly Val Pro Arg Met
 185                 190                 195                 200

AGA GAG AGA CCA ATT AGT GAT TTG GTT GAT GGT CTT AAA CAG CTT GGT      675
Arg Glu Arg Pro Ile Ser Asp Leu Val Asp Gly Leu Lys Gln Leu Gly
                 205                 210                 215

GCA GAG GTT GAT TGT TTC CTT GGT ACG AAA TGT CCT CCT GTT CGA ATT      723
Ala Glu Val Asp Cys Phe Leu Gly Thr Lys Cys Pro Pro Val Arg Ile
             220                 225                 230

GTC AGC AAG GGA GGT CTT CCT GGA GGG AAG GTC AAG CTC TCT GGA TCC      771
Val Ser Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly Ser
         235                 240                 245

ATT AGC AGC CAA TAC TTG ACT GCT CTG CTT ATG GCT GCT CCA CTG GCT      819
Ile Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu Ala
     250                 255                 260

TTA GGA GAT GTG GAG ATT GAA ATC ATT GAC AAA CTA ATT AGT GTA CCT      867
Leu Gly Asp Val Glu Ile Glu Ile Ile Asp Lys Leu Ile Ser Val Pro
 265                 270                 275                 280

TAT GTC GAG ATG ACA TTG AAG TTG ATG GAG CGA TTT GGT ATT TCT GTG      915
Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Ile Ser Val
                 285                 290                 295

GAG CAC AGT AGT AGC TGG GAC AGG TTC TTT GTC CGA GGA GGT CAG AAA      963
Glu His Ser Ser Ser Trp Asp Arg Phe Phe Val Arg Gly Gly Gln Lys
             300                 305                 310

TAC AAG TCT CCT GGA AAA GCT TTT GTC GAA GGT GAT GCT TCA AGT GCT     1011
Tyr Lys Ser Pro Gly Lys Ala Phe Val Glu Gly Asp Ala Ser Ser Ala
         315                 320                 325
```

```
AGC TAC TTC TTG GCT GGT GCA GCA GTC ACA GGT GGA ACT ATC ACT GTT      1059
Ser Tyr Phe Leu Ala Gly Ala Ala Val Thr Gly Gly Thr Ile Thr Val
    330                 335                 340

GAA GGT TGT GGG ACA AAC AGT TTA CAG GGG GAT GTC AAA TTT GCT GAG      1107
Glu Gly Cys Gly Thr Asn Ser Leu Gln Gly Asp Val Lys Phe Ala Glu
345                 350                 355                 360

GTA CTT GAA AAA ATG GGA GCT GAA GTT ACG TGG ACA GAG AAC AGT GTC      1155
Val Leu Glu Lys Met Gly Ala Glu Val Thr Trp Thr Glu Asn Ser Val
                365                 370                 375

ACA GTC AAA GGA CCT CCA AGG AGT TCT TCT GGG AGG AAG CAT TTG CGT      1203
Thr Val Lys Gly Pro Pro Arg Ser Ser Ser Gly Arg Lys His Leu Arg
            380                 385                 390

GCC ATT GAT GTG AAC ATG AAT AAA ATG CCT GAT GTT GCC ATG ACA CTT      1251
Ala Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr Leu
        395                 400                 405

GCT GTT GTT GCA CTT TAT GCT GAT GGT CCC ACA GCT ATA AGA GAT GTT      1299
Ala Val Val Ala Leu Tyr Ala Asp Gly Pro Thr Ala Ile Arg Asp Val
    410                 415                 420

GCT AGC TGG AGA GTC AAG GAA ACT GAG CGC ATG ATC GCC ATA TGC ACA      1347
Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys Thr
425                 430                 435                 440

GAA CTT AGG AAG TTA GGA GCA ACC GTT GAA GAA GGA CCA GAC TAC TGC      1395
Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr Cys
                445                 450                 455

ATA ATC ACC CCA CCG GAG AAA CTA AAT GTG ACC GAT ATT GAT ACA TAC      1443
Ile Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Asp Ile Asp Thr Tyr
            460                 465                 470

GAT GAT CAC AGG ATG GCC ATG GCT TTT TCT CTT GCT GCT TGT GCA GAT      1491
Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Ala Asp
        475                 480                 485

GTT CCC GTC ACC ATC AAT GAC CCT GGC TGC ACG CGG AAA ACC TTC CCT      1539
Val Pro Val Thr Ile Asn Asp Pro Gly Cys Thr Arg Lys Thr Phe Pro
    490                 495                 500

AAC TAC TTT GAT GTA CTT CAG CAG TAC TCC AAG CAT TGA ACCGCTTCCC      1588
Asn Tyr Phe Asp Val Leu Gln Gln Tyr Ser Lys His
505                 510                 515

TATATTGCAG AATGTAAGTA AGAATATGTG AAGAGTTTAG TTCTTGTACA AGACAGGCTA   1648

CGACTGCCTG GTATCAGAAC CACAATGGGT TCCATTTCAG TTCAGAAGGG CATTCCAAGG   1708

CTTCGAACTC TTTACTTATT TGCGAGTGAT GAAATGTATT TGTTAGAGTT GAGCTTCTTT   1768

TTGTCTTTAA GGAATGTACA CTAATAGAGT TAAGAATTAC TAGTATGGGC CAGTGTAAGG   1828

AGTACTATTA CTCTTTGCTT ATTTTATTGA TTGAGTTTTG TCAAGGATCT GGCTTTGTCA   1888

AGAATTACTG GTTAATTTTA TTGACAATCT CATGTGTCTA AATGAAATTG TTTGAT       1944
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 517 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ala Gln Ile Asn Asn Met Ala Gln Gly Ile Gln Thr Leu Asn Pro
1 5 10 15

Asn Ser Asn Phe His Lys Pro Gln Val Pro Lys Ser Ser Ser Phe Leu
20 25 30

Val Phe Gly Ser Lys Lys Leu Lys Asn Ser Ala Asn Ser Met Leu Val
35 40 45

Leu Lys Lys Asp Ser Ile Phe Met Gln Lys Phe Cys Ser Phe Arg Ile
50 55 60

Ser Ala Ser Val Ala Thr Ala Gln Lys Pro Ser Glu Ile Val Leu Gln
65 70 75 80

Pro Ile Lys Glu Ile Ser Gly Thr Val Lys Leu Pro Gly Ser Lys Ser
85 90 95

Leu Ser Asn Arg Ile Leu Leu Leu Ala Ala Leu Ser Glu Gly Thr Thr
100 105 110

Val Val Asp Asn Leu Leu Ser Ser Asp Asp Ile His Tyr Met Leu Gly
115 120 125

Ala Leu Lys Thr Leu Gly Leu His Val Glu Glu Asp Ser Ala Asn Gln
130 135 140

Arg Ala Val Val Glu Gly Cys Gly Gly Leu Phe Pro Val Gly Lys Glu
145 150 155 160

Ser Lys Glu Glu Ile Gln Leu Phe Leu Gly Asn Ala Gly Thr Ala Met
165 170 175

Arg Pro Leu Thr Ala Ala Val Thr Val Ala Gly Gly Asn Ser Arg Tyr
180 185 190

Val Leu Asp Gly Val Pro Arg Met Arg Glu Arg Pro Ile Ser Asp Leu
195 200 205

Val Asp Gly Leu Lys Gln Leu Gly Ala Glu Val Asp Cys Phe Leu Gly
210 215 220

Thr Lys Cys Pro Pro Val Arg Ile Val Ser Lys Gly Gly Leu Pro Gly
225 230 235 240

Gly Lys Val Lys Leu Ser Gly Ser Ile Ser Ser Gln Tyr Leu Thr Ala
245 250 255

Leu Leu Met Ala Ala Pro Leu Ala Leu Gly Asp Val Glu Ile Glu Ile
260 265 270

Ile Asp Lys Leu Ile Ser Val Pro Tyr Val Glu Met Thr Leu Lys Leu
275 280 285

Met Glu Arg Phe Gly Ile Ser Val Glu His Ser Ser Ser Trp Asp Arg
290 295 300

Phe Phe Val Arg Gly Gly Gln Lys Tyr Lys Ser Pro Gly Lys Ala Phe
305 310 315 320

Val Glu Gly Asp Ala Ser Ser Ala Ser Tyr Phe Leu Ala Gly Ala Ala
325 330 335

Val Thr Gly Gly Thr Ile Thr Val Glu Gly Cys Gly Thr Asn Ser Leu
340 345 350

Gln Gly Asp Val Lys Phe Ala Glu Val Leu Glu Lys Met Gly Ala Glu
355 360 365

Val Thr Trp Thr Glu Asn Ser Val Thr Val Lys Gly Pro Pro Arg Ser
370 375 380

Ser Ser Gly Arg Lys His Leu Arg Ala Ile Asp Val Asn Met Asn Lys
385 390 395 400

Met Pro Asp Val Ala Met Thr Leu Ala Val Val Ala Leu Tyr Ala Asp
405 410 415

Gly Pro Thr Ala Ile Arg Asp Val Ala Ser Trp Arg Val Lys Glu Thr
420 425 430

Glu Arg Met Ile Ala Ile Cys Thr Glu Leu Arg Lys Leu Gly Ala Thr
435 440 445

Val Glu Glu Gly Pro Asp Tyr Cys Ile Ile Thr Pro Pro Glu Lys Leu
450 455 460

Asn Val Thr Asp Ile Asp Thr Tyr Asp Asp His Arg Met Ala Met Ala
465 470 475 480

Phe Ser Leu Ala Ala Cys Ala Asp Val Pro Val Thr Ile Asn Asp Pro
485 490 495

Gly Cys Thr Arg Lys Thr Phe Pro Asn Tyr Phe Asp Val Leu Gln Gln
500 505 510

Tyr Ser Lys His
515

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Brassica napus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Brassica napus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Ala Ala Pro Leu Ala Leu Gly Asp Val Glu Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATTGAGTTGT ACCTTGGGAA TGCAGGAACA GCCATGCGTC CACTCACCGC TGCA 54

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GAGUGGACGC AUGGCUGTTG CTGCAUUCCC AAGGUACAA 39

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACTGCCCTCC TCATGGCAGC TCCTTTAGCT CTTGGAGACG TGGAGATTGA GATCATT 57

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAUCUCCACG UCUCCAAGAG TTAAAGGAGC UGCCAUGAGG A 41

(2) INFORMATION FOR SEQ ID NO: 9

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3831 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Brassica napus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AGATCTTAAA GGCTCTTTTC CAGTCTCACC TACCAAAACT ATAAGAAAAT CCACTTGCTG 60
TCTGAAATAG CCGACGTGGA TAAAGTACTT AAGACGTGGC ACATTATTAT TGGCTACTAG 120
AAAAAAAACT CATACACCAT CGTAGGAGTT GGGGTTGGTG AAGAATTTGA TGGGTGCCTC 180
TCCCCCCCCC ACTCACCAAA CTCATGTTCT TTGTAAAGCC GTCACTACAA CAACAAAGGA 240
GACGACAGTT CTATAGAAAA GCTTTCAAAT TCAATCAATG GCGCAATCTA GCAGAATCTG 300
CCATGGCGTG CAGAACCCAT GTGTTATCAT CTCCAATCTC TCCAAATCCA ACCAAAACAA 360
ATCACCTTTC TCCGTCTCCT TGAAGACGCA TCAGCCTCGA GCTTCTTCGT GGGGATTGAA 420
GAAGAGTGGA ACGATGCTAA ACGGTTCTGT AATTCGCCCG GTTAAGGTAA CAGCTTCTGT 480
TTCCACGTCC GAGAAAGCTT CAGAGATTGT GCTTCAACCA ATCAGAGAAA TCTCGGGTCT 540
CATTAAGCTA CCCGGATCCA AATCTCTCTC CAATCGGATC CTCCTTCTTG CCGCTCTATC 600
TGAGGTACAT ATACTTGCTT AGTGTTAGGC CTTTGCTGTG AGATTTTGGG AACTATAGAC 660
AATTTAGTAA GAATTTATAT ATAATTTTTT TAAAAAAAAT CAGAAGCCTA TATATATTTA 720
AATTTTTCCA AAATTTTTGG AGGTTATAGG CTTATGTTAC ACCATTCTAG TCTGCATCTT 780
TCGGTTTGAG ACTGAAGAAT TTTATTTTTT AAAAAATTAT TATAGGGAAC TACTGTAGTG 840
GACAACTTGT TGAACAGTGA TGACATCAAC TACATGCTTG ATGCGTTGAA GAAGCTGGGG 900
CTTAACGTGG AACGTGACAG TGTAAACAAC CGTGCGGTTG TTGAAGGATG CGGTGGAATA 960
TTCCCAGCTT CCTTAGATTC CAAGAGTGAT ATTGAGTTGT ACCTTGGGAA TGCAGGAACA 1020
GCCATGCGTC CACTCACCGC TGCAGTTACA GCTGCAGGTG GCAACGCGAG GTAAGGTTAA 1080
CGAGTTTTTT GTTATTGTCA AGAAATTGAT CTTGTGTTTG ATGCTTTTAG TTTGGTTTGT 1140
TTTCTAGTTA TGTACTTGAT GGGGTGCCTA GAATGAGGGA AAGACCTATA GGAGATTTGG 1200
TTGTTGGTCT TAAGCAGCTT GGTGCTGATG TTGAGTGTAC TCTTGGCACT AACTGTCCTC 1260
CTGTTCGTGT CAATGCTAAT GGTGGCCTTC CCGGTGGAAA GGTGATCTTC ACATTTACTC 1320
TATGAATTGT TTGCAGCAGT CTTTGTTCAT CACAGCCTTT GCTTCACATT ATTTCATCTT 1380
TTAGTTTGTT GTTATATTAC TTGATGGATC TTTAAAAAGG AATTGGGTCT GGTGTGAAAG 1440
TGATTAGCAA TCTTTCTCGA TTCCTTGCAG GGCCGTGGGC ATTACTAAGT GAAACATTAG 1500
CCTATTAACC CCCAAAATTT TTGAAAAAAA TTTAGTATAT GGCCCCAAAA TAGTTTTTTA 1560
AAAAATTAGA AAAACTTTTA ATAAATCGTC TACAGTCCCN NAAATCTTAG AGCCGGCCCT 1620
GCTTGTATGG TTTCTCGATT GATATATTAG ACTATGTTTT GAATTTTCAG GTGAAGCTTT 1680
CTGGATCGAT CAGTAGTCAG TACTTGACTG CCCTCCTCAT GGCAGCTCCT TTAGCTCTTG 1740
GAGACGTGGA GATTGAGATC ATTGATAAAC TGATATCTGT TCCATATGTT GAAATGACAT 1800

TGAAGTTGAT GGAGCGTTTT GGTGTTAGTG CCGAGCATAG TGATAGCTGG GATCGTTTCT 1860

TTGTCAAGGG CGGTCAGAAA TACAAGTAAT GAGTTCTTTT AAGTTGAGAG TTAGATTGAA 1920

GAATGAATGA CTGATTAACC AAATGGCAAA ACTGATTCAG GTCGCCTGGT AATGCTTATG 1980

TAGAAGGTGA TGCTTCTAGT GCTAGCTATT TCTTGGCTGG TGCTGCCATT ACTGGTGAAA 2040

CTGTTACTGT CGAAGGTTGT GGAACAACTA GCCTCCAGGT AGTTTATCCA CTCTGAATCA 2100

TCAAATATTA TTCTCCCTCC GTTTTATGTT AAGTGTCATT AGCTTTTAAA TTTTGTTTCA 2160

TTAAAAGTGT CATTTTACAT TTTCAATGCA TATATTAAAT AAATTTTCCA GTTTTTACTA 2220

ATTCATTAAT TAGCAAAATC AAACAAAAAT TATATTAAAT AATGTAAAAT TCGTAATTTG 2280

TGTGCAAATA CCTTAAACCT TATGAAACGG AAACCTTATG AAACAGAGGG AGTACTAATT 2340

TTATAATAAA ATTTGATTAG TTCAAAGTTG TGTATAACAT GTTTTGTAAG AATCTAAGCT 2400

CATTCTCTTT TTATTTTTTG TGATGAATCC AAAGGGAGAT GTGAAATTCG CAGAGGTTCT 2460

TGAGAAAATG GGATGTAAAG TGTCATGGAC AGAGAACAGT GTGACTGTGA CTGGACCATC 2520

AAGAGATGCT TTTGGAATGA GGCACTTGCG TGCTGTTGAT GTCAACATGA ACAAAATGCC 2580

TGATGTAGCC ATGACTCTAG CCGTTGTTGC TCTCTTTGCC GATGGTCCAA CCACCATCAG 2640

AGATGGTAAA GCAAAACCCT CTCTTTGAAT CAGCGTGTTT TAAAAGATTC ATGGTTGCTT 2700

AAACTCTATT TGGTCAATGT AGTGGCTAGC TGGAGAGTTA AGGAGACAGA GAGGATGATT 2760

GCCATTTGCA CAGAGCTTAG AAAGGTAAGT TTCCTTTTCT CTCATGCTCT CTCATTCGAA 2820

GTTAATCGTT GCATAACTTT TTGCGGTTTT TTTTTTTGCG TTCAGCTTGG AGCTACAGTG 2880

GAAGAAGGTT CAGATTATTG TGTGATAACT CCACCAGCAA AGGTGAAACC GGCGGAGATT 2940

GATACGTATG ATGATCATAG AATGGCGATG GCGTTCTCGC TTGCAGCTTG TGCTGATGTT 3000

CCAGTCACCA TCAAGGATCC TGGCTGCACC AGGAAGACTT TCCCTGACTA CTTCCAAGTC 3060

CTTGAAAGTA TCACAAAGCA TTAAAAGACC CTTTCCTCTG ATCCAAATGT GAGAATCTGT 3120

TGCTTTCTCT TTGTTGCCAC TGTAACATTT ATTAGAAGAA CAAAGTGTGT GTGTTAAGAG 3180

TGTGTTTGCT TGTAATGAAC TGAGTGAGAT GCAATCGTTG AATCAGTTTT GGGCCTTAAT 3240

AAAGGGTTTA GGAAGCTGCA GCGAGATGAT TGTTTTTGAT CGATCATCTT TGAAAATGTG 3300

TTTGTTTGAG TAATTTTTCT AGGGTTGAGT TGATTACACT AAGAAACACT TTTTGATTTT 3360

CTATTACACC TATAGACACT TCTTACATGT GACACACTTT GTTGTTGGCA AGCAACAGAT 3420

TGTGGACAAT TTTGCCTTTA ATGGAAAGAA CACAGTTGTG GATGGGTGAT TTGTGGACGA 3480

TTCCATGTGT GGTTAGGGTG ATTTGTGGAC GGATGATGTG TAGATGAGTG ATGAGTAATG 3540

TGTGAATATG TGATGTTAAT GTGTTTATAG TAGATAAGTG GACAAACTCT CTGTTTTGAT 3600

TCCATAAAAC TATACAACAA TACGTGGACA TGGACTCATG TTACTAAAAT TATACCGTAA 3660

AACGTGGACA CGGACTCTGT ATCTCCAATA CAAACACTTG GCTTCTTCAG CTCAATTGAT 3720

AAATTATCTG CAGTTAAACT TCAATCAAGA TGAGAAAGAG ATGATATTGT GAATATGAGC 3780

GGAGAGAGAA ATCGAAGAAG CGTTTACCTT TTGTCGGAGA GTAATAGATC T 3831

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 516 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Brassica napus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Ala Gln Ser Ser Arg Ile Cys His Gly Val Gln Asn Pro Cys Val
1 5 10 15

Ile Ile Ser Asn Leu Ser Lys Ser Asn Gln Asn Lys Ser Pro Phe Ser
20 25 30

Val Ser Leu Lys Thr His Gln Pro Arg Ala Ser Ser Trp Gly Leu Lys
35 40 45

Lys Ser Gly Thr Met Leu Asn Gly Ser Val Ile Arg Pro Val Lys Val
50 55 60

Thr Ala Ser Val Ser Thr Ser Glu Lys Ala Ser Glu Ile Val Leu Gln
65 70 75 80

Pro Ile Arg Glu Ile Ser Gly Leu Ile Lys Leu Pro Gly Ser Lys Ser
85 90 95

Leu Ser Asn Arg Ile Leu Leu Leu Ala Ala Leu Ser Glu Gly Thr Thr
100 105 110

Val Val Asp Asn Leu Leu Asn Ser Asp Asp Ile Asn Tyr Met Leu Asp
115 120 125

Ala Leu Lys Lys Leu Gly Leu Asn Val Glu Arg Asp Ser Val Asn Asn
130 135 140

Arg Ala Val Val Glu Gly Cys Gly Gly Ile Phe Pro Ala Ser Leu Asp
145 150 155 160

Ser Lys Ser Asp Ile Glu Leu Tyr Leu Gly Asn Ala Gly Thr Ala Met
165 170 175

Arg Pro Leu Thr Ala Ala Val Thr Ala Ala Gly Gly Asn Ala Ser Tyr
180 185 190

Val Leu Asp Gly Val Pro Arg Met Arg Glu Arg Pro Ile Gly Asp Leu
195 200 205

Val Val Gly Leu Lys Gln Leu Gly Ala Asp Val Glu Cys Thr Leu Gly
210 215 220

Thr Asn Cys Pro Pro Val Arg Val Asn Ala Asn Gly Gly Leu Pro Gly
225 230 235 240

Gly Lys Val Lys Leu Ser Gly Ser Ile Ser Ser Gln Tyr Leu Thr Ala
245 250 255

Leu Leu Met Ala Ala Pro Leu Ala Leu Gly Asp Val Glu Ile Glu Ile
260 265 270

Ile Asp Lys Leu Ile Ser Val Pro Tyr Val Glu Met Thr Leu Lys Leu
275 280 285

Met Glu Arg Phe Gly Val Ser Ala Glu His Ser Asp Ser Trp Asp Arg
290 295 300

Phe Phe Val Lys Gly Gly Gln Lys Tyr Lys Ser Pro Gly Asn Ala Tyr
305 310 315 320

Val Glu Gly Asp Ala Ser Ser Ala Ser Tyr Phe Leu Ala Gly Ala Ala
325 330 335

Ile Thr Gly Glu Thr Val Thr Val Glu Gly Cys Gly Thr Thr Ser Leu
340 345 350

Gln Gly Asp Val Lys Phe Ala Glu Val Leu Glu Lys Met Gly Cys Lys
355 360 365

Val Ser Trp Thr Glu Asn Ser Val Thr Val Thr Gly Pro Ser Arg Asp
370 375 380

Ala Phe Gly Met Arg His Leu Arg Ala Val Asp Val Asn Met Asn Lys
385 390 395 400

Met Pro Asp Val Ala Met Thr Leu Ala Val Val Ala Leu Phe Ala Asp
405 410 415

Gly Pro Thr Thr Ile Arg Asp Val Ala Ser Trp Arg Val Lys Glu Thr
420 425 430

Glu Arg Met Ile Ala Ile Cys Thr Glu Leu Arg Lys Leu Gly Ala Thr
435 440 445

Val Glu Glu Gly Ser Asp Tyr Cys Val Ile Thr Pro Pro Ala Lys Val
450 455 460

Lys Pro Ala Glu Ile Asp Thr Tyr Asp Asp His Arg Met Ala Met Ala
465 470 475 480

Phe Ser Leu Ala Ala Cys Ala Asp Val Pro Val Thr Ile Lys Asp Pro
485 490 495

Gly Cys Thr Arg Lys Thr Phe Pro Asp Tyr Phe Gln Val Leu Glu Ser
500 505 510

Ile Thr Lys His
515

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTATGATCCC TAATGGTGGG GCTTTTTTAA GCCCACCATT AGGGAUCAUA GGCGCGTTTT 60

CGCGC 65

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTAATGCAGG AATAGCAATG CGTCCTTTTG GACGCAUUGC TATTCCUGCA UUACGCGCGT 60

TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GTAATGCAGG AATAGCAATG CGTTCTTTTG AACGCAUUGC TATTCCTGCA UUACGCGCGT 60

TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ACAGCAATGC GTTCACTTAC CGCTGTTTTC AGCGGUAAGT GAACGCAUUG CUGUGCGCGT 60

TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ATAGCAATGC GTTCACTTAC CGCTGTTTTC AGCGGUAAGT GAACGCAUUG CUAUGCGCGT 60

TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GTAATGCAGG AATAGCAATG CGTTCACTCA CCGCTGTTTT CAGCGGUGAG TGAACGCAUU 60

GCTATTCCUG CAUUACGCGC GTTTCGCGC 89

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GGAATGCAGG AATAGCCATG CGTCCTTTTG GACGCAUCGC TATTCCUGCA UUCCGCGCGT 60

TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GGAATGCAGG AATAGCCATG CGTTCTTTTG AACGCAUCGC TATTCCTGCA UUCCGCGCGT 60

TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

ACAGCCATGC GTTCACTCAC CGCTGTTTTC AGCGGUGAGT GAACGCAUGG CUGUGCGCGT 60

TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ATAGCCATGC GTTCACTCAC CGCTGTTTTC AGCGGUGAGT GAACGCAUGG CUAUGCGCGT 60

TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGAATGCAGG AATAGCCATG CGTTCACTCA CCGCTGTTTT CAGCGGUGAG TGAACGCAUC 60

GCTATTCCUG CAUUCCGCGC GTTTCGCGC 89

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGAATGCTGG AATCGCAATG CGGCCATTTT TAUGGCCGCA UUGCGATTCC AGCAUUCCGC 60

GCGTTTCGCG C 71

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GGAATGCTGG AATCGCAATG CGGTCATTTT TAUGACCGCA UUGCGATTCC AGCAUUCCGC 60

GCGTTTCGCG C 71

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CTGCAATGCG GTCATTGACA GCAGCTTTTG CUGCUGUCAA TGACCGCAUU GGCAGGCGCG 68

TTTCGCGC

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

TCGCAATGCG GTCATTGACA GCAGCTTTTG CUGCTGUCAA TGACCGCAUU GCGAGCGCGT 60

TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GGAATGCTGG AATCGCAATG CGGTCATTGA CAGCAGCTTT TGCUGCUGUC AATGACCGCA 60

UUGCGATTCC AGCAUUCCGC GCGTTTCGCG C 91

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

TCGCATTGAA CAGCTTTCTT CAGGTTTTTA CCUGAAGAAA GCTGUUCAAU GCGAGCGCGT 60

TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

TTGTACCTTG GGAATGCAGG AACAGCCATG CGTCCACTC 39

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

TCCTCATGGC AGCTCCTTTA GCTCTTGGAG ACGTGGAGAT T 41

## CLAIMS

1. A method of producing plants which exhibit an agronomically desirable trait comprising mutating or otherwise modifying *in situ* in a plant cell at least one gene which when modified is responsible for providing the said trait and regenerating from a cell exhibiting the said trait fertile morphologically normal whole plants, characterised in that a polynucleotide is introduced into the plant cell, the said polynucleotide comprising at least one region which is substantially complementary to at least one region in the gene, which gene region when mutated or otherwise modified provides for the agronomically desirable trait, the region in the said polynucleotide containing at least one base mismatch in comparison with the like region in the said gene, so that the region in the said gene is altered by the DNA repair/replication system of the cell to include the said mismatch.

2. A method according to the preceding claim, wherein - prior to the *in situ* mutation or modification, the plant cell is transformed with a gene providing for an agronomically desirable trait, and/or the cell is treated with a chemical mutagen.

3. A method according to either of claims 1 or 2, wherein at least one of the following regions of the gene is mutated or otherwise modified: promoter, RNA encoding sequence or transcription terminator.

4. A method according to any preceding claim, wherein the transcription activating region of the gene is mutated or otherwise modified *in situ*.

5. A method according to any preceding claim, wherein the said trait is herbicide resistance.

6. A method according to the preceding claim, wherein the herbicide is selected from the group consisting of paraquat; glyphosate; glufosinate; photosystem II inhibiting herbicides; dinitroanaline or other tubulin binding herbicides; herbicides which inhibit imidazole glycerol phosphate dehydratase; herbicides which inhibit

acetolactate synthase; herbicides which inhibit acetyl CoA carboxylase; herbicides which inhibit protoporphyrinogen oxidase; herbicides which inhibit phytoene desaturase; herbicides which inhibit hydroxyphenylpyruvate dioxygenase and herbicides which inhibit the biosynthesis of cellulose.

7. A method according to any one of claims 2 to 6, wherein the plant cell is prior transformed with a gene providing for resistance to insects, fungi, and/or herbicides.

8. A method according to any preceding claim, wherein the protein encoding region of the gene encodes an enzyme selected from the group consisting of EPSPS, GOX, PAT, HPPD, ACC, ALS, BNX and protox.

9. A method according to the preceding claim, wherein the said at least one region of the polynucleotide consists of RNA.

10. A method according to the preceding claim, wherein the polynucleotide other than that comprised by the said at least one region consists of DNA.

11. A method according to any one of the preceding claims, wherein the polynucleotide consists of between about 30 and 250 nucleotides.

12. A method according to the preceding claim, wherein the polynucleotide consists of between 50 and 80 nucleotides.

13. A method according to any preceding claim, wherein the polynucleotide comprises between about 60 and about 150 bases and has an overall 'dumbbell' like shaped secondary structure looped around upon itself at either end and with a central 'rod' region of paired complementary DNA and RNA sequences.

14. A method according to any one of claims 8 to 13, in which the said gene encodes an EPSPS having at least the residues Thr, Pro, Gly and Ala at positions corresponding to 174, 178, 173 and 264 with respect to the EPSPS depicted in SEQ ID No. 2,

wherein the said mismatch results in at least one of the following modifications in the EPSPS enzyme in comparison with the native sequence:

(i) Thr 174 - Ile

(ii) Pro 178 - Ser

(iii) Gly 173 - Ala

(iv) Ala 264 - Thr

wherein (i) Thr 174 occurs within a sequence comprising contiguously Ala -Gly-Thr-Ala-Met; (ii) Pro 178 occurs within a sequence comprising contiguously Met-Arg-Pro-Leu-Thr; (iii) Gly 173 occurs within a sequence comprising contiguously Asn-Ala-Gly-Thr-Ala; and (iv) Ala 264 occurs within a sequence comprising contiguously Pro-Leu-Ala-Leu-Gly.

15. A method according to any one of claims 8 to 14, wherein the mismatch results in replacement of the terminal Gly residue within the sequence motif Glu-Arg-Pro-AA1-AA2-AA3-Leu-Val-AA4-AA5-Leu-AA6-AA7-AA8-Gly- in a region of the EPSPS enzyme corresponding to that spanning positions 202 to 216 in SEQ ID No. 2 by either an Asp or Asn residue.

16. A method according to any preceding claim, wherein the plant cell is a cell of a plant selected from the group consisting of canola, sunflower, tobacco, sugar beet, cotton, maize, wheat, barley, rice, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, cabbage, onion, soya spp, sugar cane, pea, field beans, poplar, grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax and oilseed rape, and nut producing plants insofar as they are not already specifically mentioned.

17. A method according to any preceding claim, wherein the plant cell is converted into a protoplast prior to the *in situ* mutation or modification of the gene, or transcriptional activating regions thereof, which when modified provides for the agronomically desirable trait.

18. Plants which result from the method of any preceding claim, the progeny and seeds of such plants, and plant material derived from such plants, progeny and seeds.

19. A method of controlling weeds in a field, the field comprising weeds and plants according to claim 18, the method comprising application to the field of a herbicide to which the said plants have been rendered resistant.

20. A method according to the preceding claim, further comprising the steps of applying to the field insecticidally effective amounts of insecticides and/or fungicidally effective amounts of fungicides after the field has been treated with the herbicide.

# INTERNATIONAL SEARCH REPORT

| | |
|---|---|
| International Application No | PCT/GB 98/01499 |

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N15/82 C12N15/90 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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| X<br>A | WO 91 04323 A (MONSANTO CO) 4 April 1991<br>* see esp. p.4-10 *<br>--- | 18,19<br>5-17,20 |
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| X<br><br>A | WO 97 04103 A (RHONE POULENC AGROCHIMIE ;LEBRUN MICHEL (FR); SAILLAND ALAIN (FR);) 6 February 1997<br>* see esp. p.10 *<br>---<br>-/-- | 18,19<br><br>5-17,20 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 26 August 1998 | 02/09/1998 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Kania, T |

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# INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01499

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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